PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

STEINECKE, Peter
Müller Fottner Steinecke
P.O. Box 31 01 40
80102 München
ALLEMAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	23.06.2005
Applicant's or agent's file reference AX02A15/P-WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2004/007530	08.07.2004	08.07.2003

Applicant
AXIOGENESIS AG et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary report on patentability and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary report on patentability. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

The applicant's attention is drawn to Article 33(5), which provides that the criteria of novelty, inventive step and industrial applicability described in Article 33(2) to (4) merely serve the purposes of international preliminary examination and that "any Contracting State may apply additional or different criteria for the purposes of deciding whether, in that State, the claimed inventions is patentable or not" (see also Article 27(5)). Such additional criteria may relate, for example, to exemptions from patentability, requirements for enabling disclosure, clarity and support for the claims.

Name and mailing address of the international preliminary examining authority:	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Pepper Cano, E Tel. +49 89 2399-5636

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference AX02A15/P-WO	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/EP2004/007530	International filing date *(day/month/year)* 08.07.2004	Priority date *(day/month/year)* 08.07.2003
International Patent Classification (IPC) or national classification and IPC C12N5/06		
Applicant AXIOGENESIS AG et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 6 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 5 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☒	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09.05.2005	23.06.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Friedrich, C Telephone No. +49 89 2399-7721

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/007530

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages	
1-44	as originally filed
Claims, Numbers	
1-42	received on 09.05.2005 with letter of 09.05.2005
Drawings, Sheets	
1/6-6/6	as originally filed

   ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☒ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☒ the claims, Nos. 43,44
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2004/007530

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation to restrict or pay additional fees, the applicant has:
   - ☒ restricted the claims.
   - ☐ paid additional fees.
   - ☐ paid additional fees under protest.
   - ☐ neither restricted nor paid additional fees.
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
   - ☐ complied with.
   - ☐ not complied with for the following reasons:
4. Consequently, this report has been established in respect of the following parts of the international application:
   - ☒ all parts.
   - ☐ the parts relating to claims Nos. .

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-42
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-42
Industrial applicability (IA)	Yes:	Claims	1-42
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

The following documents (D) are referred to in this communication:

D1: US 2003/119107 A1 (DANG STEPHEN ET AL) 26 June 2003.
D2: WO 01 62899 A (WISCONSIN ALUMNI RES FOUND) 30 August 2001.

Introduction

The gist of the present application appears to be the production of embryoid bodies (EBs) from pluriopotent cells, where a high concentration liquid suspension cell culture is agitated until formation of aggregates.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Novelty, Art.33(1) and (2), PCT

Subject-matter referred to in claims 1-42 has not been disclosed in the prior art and appears to be novel under Art.33 (2), PCT.

Schwerpunkt / Hauptanspruch

2. Inventive Step, Art.33(1) and (3), PCT

2.1. The gist of the present application appears to be agitation rather than stirring of liquid suspension cultures for the formation of embryoid bodies (EBs) from multi- or pluripotent cells (page 9 of the description). According to examples 1 and 2 agitation is achieved with rocking tables. Document D1 in which the importance of controlling cell aggregation during formation of EBs from ES cells is disclosed (see paragraph 0054) is considered the closest prior art. It is stated in paragraph 0054 that aggregation sufficient to induce spheroid formation is permitted but aggregation beyond that and aggregation between separate EBs is prevented. A cell concentration of 10^6 cells/ml (paragraph 0050) and agitation of the culture system as one means of controlling aggregation (paragraph 0053) are specifically disclosed. In D1, however, no advantage of agitation over stirring or any other method or means of controlling aggregation is disclosed. From this subject-matter of the present application differs in that agitation is superior over other means of aggregation control. The technical problem thus appears to be the improvement of aggregation control in cultures of EB formation. Since the

present application does not disclose any advantageous effects of agitation over other means of aggregation control (comparative experiments are missing), the choice of agitation appears to be a simple selection from well known possibilities, without the provision of any surprising effects and obvious to the skilled person in the art. Since the description does not indicate whether the proposed agitation in fact improves EB formation over that achieved by other means of aggregation control, the objective technical problem does not appear to be solved. Therefore subject-matter referred to in claims 1-7 does not appear to involve an inventive step under Art. 33 (3), PCT.

2.2. Claims 8-42 refer to standard culture conditions, standard cell differentiation protocols, and kits the composition of which is simply based on said methods. Said claims do not appear to contain any additional features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT with respect to novelty and/or inventive step.

3. Industrial Applicability, Art.33 (1) and (4), PCT

Subject-matter of the present application appears to be industrially applicable under Art.33(1) and (4), PCT.

Re Item VII

Certain defects in the international application

Disclosure of the Invention, Art.5, PCT

Independent claim 1 of the present application refers to the production of EBs from multi- or pluriopotent cells, including ES cells, EG cells, or adult somatic stem cells, without further defining the species said cells are derived from. It is implied that agitation of a liquid culture as referred to in claim 1 and demonstrated for mouse ES cells in examples 1-2 has the same effect on all multi- or pluriopotent cells. In this respect applicant's attention is drawn to D1, page 3 which indicates that conventional murine culture protocols fail e.g. for primate cells. Consequently, only the formation of EBs from murine ES cells, as shown in examples 1 and 2 is considered to be sufficiently disclosed under Art.5, PCT. Furthermore, the Examination Authority is not aware of protocols for the formation of EBs, e.g. from adult somatic stem cells such as haematopoietic or neuronal stem cells.

? D2

2. Exceptions to Patentability

It is pointed out to the applicant that upon entry into the regional phase certain subject-matter claimed in the present application is not patentable. The EPO, for example, does not does not recognize as patentable subject-matter relating to the use of human embryos for commercial purposes.

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PCT/EP2004/007530
Axiogenesis AG
Our Ref.: AX02A15/P-WO

Claims

1. A method for producing embryoid bodies (EBs) from multi- or pluripotent cells comprising
   (a) agitation of a liquid suspension culture of multi- or pluripotent cells in a container until generation of cell aggregates; and
   (b) optionally diluting the suspension, and further agitation of the suspension until formation of EBs.

2. The method of claim 1, wherein prior to step (a) the cells are cultured on embryonic mouse fibroblasts (feeder cells).

3. The method of claim 1 or 2, wherein said multi- or pluripotent cells are embryonic stem (ES) cells.

4. The method of any one of claims 1 to 3, wherein said cells are derived from a murine ES cell line.

5. The method of any one of claims 1 to 4, wherein the culture medium in step (a) and/or (b) is IMDM 20 % FCS and 5 % CO_2.

6. The method of any one of claims 1 to 5, wherein the culture conditions in step (a) and/or (b) comprise 37 °C and 95 % humidity.

7. The method of any one of claims 1 to 6, wherein said culture of multi- or pluripotent cells has a concentration of about $1x\ 10^6$ to $5x\ 10^6$ cells/ml.

8. The method of claim 7, wherein the suspension in step (a) is cultured for about 6 hours.

9. The method of claim 7 or 8, wherein the suspension in step (b) is cultured for about 16 to 20 hours.

10. The method of any one of claims 7 to 9, wherein the suspension in step (b) is cultured in T25 flasks.

11. The method of any one of claims 1 to 10, wherein said dilution in step (b) is 1:10.

12. The method of any one of claims 1 to 11, wherein the final concentration of EBs in the suspension culture is about 500/ml.

13. The method of any one of claims 1 to 12, further comprising dividing the cell aggregates to the desired final concentration.

14. The method of any one of claims 1 to 6, wherein said culture of multi- or pluripotent cells has a concentration of about $0.1x10^6$ to $0.5x10^6$ cells/ml.

15. The method of claim 14, wherein the suspension is cultured for about 48 hours.

16. The method of claim 14 or 15, wherein the resultant EBs are diluted to a concentration of about 100-2000 EBs/10 ml.

17. The method of any one of claims 1 to 16, further comprising culturing the cells under conditions allowing differentiation of the cells into at least one cell type.

18. The method of claim 17, wherein said cell type is selected from cardiomyocytes, neurons, endothelial cells, hepatocytes, fibroblasts, skeletal muscle cells, smooth muscle cells and chondrocytes.

19. The method of any one of claims 1 to 16, further comprising selection of desired cell types by use of one or more selectable markers and/or agents.

20. The method of any one of claims 1 to 19, wherein said cell is genetically engineered.

21. The method of any one of claims 1 to 20, wherein said cell comprises a selectable marker and/or a reporter gene.

22. The method of any one of claims 1 to 21, wherein said cell comprises a selectable marker gene operably linked to a cell type-specific regulatory sequence.

23. The method of claim 22, wherein said selectable marker confers resistance to puromycin.

24. The method of any one of claims 1 to 23, wherein said cell comprises a reporter gene operably linked to a cell type-specific regulatory sequence.

25. The method of claim 24, wherein said cell type-specific regulatory sequence of the reporter gene is substantially the same as said cell type-specific regulatory sequence of the marker gene.

26. The method of claim 25, wherein said reporter is selected from different color versions of enhanced green fluorescent protein (EGFP).

27. The method of any one of claims 22 to 26, wherein said marker gene and said reporter gene are contained on the same recombinant nucleic acid molecule.

28. The method of claim 27, wherein said marker gene and said reporter gene are contained on the same cistron.

29. The method of any one of claims 22 to 28, wherein said cell type-specific regulatory sequence is atrial- and/or ventricular-specific.

30. The method of claim 29, wherein said regulatory sequence is a cardiac-specific regulatory sequence selected from promoters of αMHC or MLC2v.

31. A method of producing a differentiated cell or tissue derived from an embryoid body comprising the method of any one of claims 1 to 30.

32. The method of claim 31, wherein the cell is a cardiomyocyte.

33. A method for identifying and/or obtaining a drug or for determining the toxicity of a compound comprising the steps of the method for producing an embryoid body (EB) of any one of claims 1 to 32, and further comprising:

(a) contacting a test sample comprising said embryoid body (EB) with a test substance to be screened; and

(b) determining the effect of the test substance on the EB or on the amount of the reporter gene product or activity compared to a control sample.

34. The method of claim 33, wherein said effect on the EB is a characteristic of the differentiated cell.

35. The method of claim 33 or 34, wherein said method is performed on a microwell plate or an array.

36. The method of claim 35, wherein said array is a microelectrode array (MEA).

37. The method of any one of claims 33 to 36, wherein said embryoid body consists of cardiac cells.

38. The method of any one of claims 33 to 37, comprising determining the fluorescence of said embryoid body.

39. The method of any one of claims 33 to 38 comprising:

(i) determining the amount of cardiac cells within the embryoid body by measurement of fluorescence;

(ii) measurement of cardiac-specific characteristics; and optionally

(iii) measurement of cell viability and/or apoptotic events.

40. Use of the method of any one of claims 1 to 32 for loss of function assays of specific genes, gain of function assays of exogenous genes, developmental analysis of teratogenic/embryotoxic compounds, pharmacological assays, microarray systems, establishment of model systems for pathological cell functions, application of differentiation and growth factors for induction of selectively differentiated cells, as a

source for tissue grafts, or for the manufacture of a pharmaceutical composition comprising an embryoid body or a differentiated cell or a tissue derived therefrom.

41. Kit for use in a method of any one of claims 1 to 39 comprising culture media components, selectable markers, reference samples, microarrays, vectors, probes, containers, or multi- or pluripotent cells.

42. Use of a cell container, devices for agitation and/or culturing cells, culture media and components thereof, multi- or pluripotent cells, vectors, fluorescence reader, or microscope or a microarray for a method of any one of claims 1 to 39.